The Involvement of Membrane-Degrading Enzymes During Infection of Potato Leaves by *Phytophthora infestans*

During the infection of potato leaves by Phytophthora infestans there is a rapid degradation of membrane components (chlorophyll, galactolipids, and phospholipids). We have identified an inducible phospholipase B activity which is secreted by the fungus in culture and also occurs during the infection of potato leaves. Under conditions which result in maximal induction of phospholipase activity in culture filtrates, comparable levels of galactolipase and triacylglycerol lipase are also observed. During the infection of potato leaves there was a large (>14-fold) increase in total phospholipase activity. Since potato leaves also contain very high levels of phospholipase and galactolipase activities, experiments were conducted to elucidate the possible involvement of the lipolytic enzymes of the pathogen and the host during infection and resistance.

Phytophthora infestans is the fungal pathogen that causes late blight of potatoes. The Irish "potato famine" of the 1840's was caused by P. infestans. Even today, late blight is still the single most important disease of potatoes worldwide (1). With modern farming techniques approximately 10% of the world potato crop and 4% of the U.S. potato crop are lost in the field to this disease each year (2,3). In addition to causing these large losses in the field, late blight and the secondary bacterial infections which often accompany it cause comparable losses during the storage of tubers.

Despite many years of intensive breeding research, no existing cultivars of European or North American potatoes allow commercial cultivation in humid regions without fungicide protection (1). At best, farmers can choose cultivars with a moderate level of general resistance (i.e., Sebago) which are protected by fewer applications

of fungicide than are required by other cultivars. Our laboratory is interested in studying disease resistance mechanisms at the subcellular level, especially at the membrane level. For our preliminary studies we have chosen to study the changes in membrane lipid composition that occur during a susceptible host-pathogen interaction. We have attempted to correlate changes in lipid composition with changes in the levels of lipolytic enzymes of the host and pathogen separately and during infection. This report will summarize our recent studies (4-7) of the P. infestans-potato leaf interaction and compare them with comparable studies of other host-pathogen interactions.

Changes in Host Ultrastructure and Lipid Composition during Infection

In the field, infection of potato plants by P. infestans is usually localized in the leaves. Encysted zoospores germinate on the leaf surface, form an appressorium, and penetrate the cuticular layers of the leaf. The recent work of Wilson and Coffey (8) indicates that direct penetration of epidermal cells adjacent to stomatal cells is the most common mode of entry. Hyphae spread both intra- and intercellularly forming haustoria when host cells are penetrated (9,10). In susceptible interactions, host cells contain "organelles the structure of which is disorganized" (10) or "very disintegrated organelles" (9). In contrast, fungal organelles remain intact long after the surrounding host tissue has been disrupted (10). In a resistant interaction the fungus actively infects the leaves for the first 9-12 h but is then subsequently killed by 24 h (9).

Unfortunately, no histochemical or cytochemical studies of the involvement of lipolytic enzymes during the infection of potato leaves by P. infestans have yet been reported. Histochemical and biochemical observations of potato tubers infected by P. infestans revealed elevated levels of esterase (measured with α-naphthol acetate) in unidentified host organelles during infection (11). Cytochemical techniques were used to identify lipolytic activity in fungal and host cell walls during the infection of lettuce (Lactuca sativa L.) cotyledons by Bremia lactucae (12).

Very little is known about the changes that occur in the composition of membrane lipids during the infection of plants by fungal pathogens. Three such studies have been reported (13-15) and deal with the infection of susceptible leaves by rust species. In each case there was a rapid disappearance of chloroplast glycolipids and phosphatidyl glycerol, and evidence for the presence of fungal phospholipids during latter stages of infection. During the infection of bean plants (Phaseolus vulgaris) by Uromyces phaseoli, there was an increase in the proportion of unsaturated fatty acids and this change was attributed to fungal growth (16).

In our preliminary studies of the infection of susceptible potato leaves by P. infestans we also observed a similar rapid degradation of glycolipids and phosphatidyl glycerol. However, upon further investigation we realized that the unique composition of membrane lipids in P. infestans may provide a useful marker during infection. The polar lipid composition of healthy potato leaves and cultured P. infestans is shown in Table I. The lipid

Table I. Polar Lipid Composition of Potato Leaves and P. infestans. Fungal cultures were grown in liquid French bean media (4) for 14 days at 14° without shaking. Lipids were extracted (42) separated by 2-dimensional TLC (18). Individual spots were identified by comparison with standards, scraped from TLC plates, and analyzed for phosphorous (6) and hexose (43).

	mole %	
Lipid Class[a]	Healthy Potato Leaf	P. infestans
MGDG	53	0
DGDG	20	0
SQD	3	0
PC	14	46
PG	3	0
PE	6	39
PI	1	2
CAEP	0	13

[a] Abbreviations: MGDG = monogalactosyldiglyceride, DGDG = digalactosyldiglyceride, SQD = sulfoquinovasyldiglyceride, PC = phosphatidylcholine, PG = phosphatidylglycerol, PE = phosphatidylethanolamine, PI = phosphatidylinositol, CAEP = ceramide aminoethylphosphonate.

composition of potato leaves is comparable to that of the leaves of other species of angiosperms (17). P. infestans contains high levels of phosphatidylcholine and phosphatidylethanolamine, as is common for fungi (13), but it also contains an unusual sphingolipid, ceramide aminoethylphosphonate (CAEP) (Figure 1). This sphingolipid was previously identified in two closely related fungi Pythium prolatum and Phytophthora parasitica var. nicotianae (18). We have recently observed that after 6 days of infection CAEP is detectable in thin layer chromatograms of lipids from infected leaves. Since the fungal ceramide lacks carboxyl ester bonds it is resistant to hydrolysis by phospholipase B and its presence may render certain fungal membranes less vulnerable to degradation during infection. P. infestans also contains high levels of two unusual fatty acids, arachidonic acid (20:4) and eicosapentaenoic acid (20:5) (19) which are absent in the potato plant and could also be used as markers of fungal lipids during infection. We are currently studying the quantitative changes in these membrane lipid components during infection.

Properties of Phospholipases from Phytopathogens

Phospholipases are enzymes that catalyze the hydrolysis of membrane phospholipids. There are six types of phospholipases (A_1, A_2, B, C, D, and lysophospholipase). Each hydrolyses a different part of the phospholipid molecule and results in the formation of different

products (Figure 2). Although some assay techniques are capable of identifying the type of phospholipase activity, several of the common techniques only measure total breakdown of phospholipid. Phospholipase activity has been reported to occur in eleven phytopathogens (ten fungi and one bacteria) (4,16,20-27) (Table II). In six of these species, phospholipase B (which hydrolyzes two fatty acids per phospholipid molecule) was detected. Only three of these studies (20,21,22) have reported the effect of fungal phospholipases on plant tissue. In Table II, it is noted whether these enzyme activities were detected in fungal cultures (intracellular or extracellular), or in infected plant tissue. The pH optimum for each enzyme is also listed.

Table II. Occurrence of Phospholipase Activity in Phytopathogens

Species	Type of Phospholipase	Optimum pH	Localization[a] of Enzyme	Ref.
Botrytis cinerea	B	5.0	M	21
Erwinia carotovora	C	Assayed only at 8.0	E	22
Erysiphe pisi	A_2	Assayed only at 8.9	I	20
Fusarium solani	?	4.0	E	23
Phoma medicaginis	B	?	M	24
Phytophthora infestans	B	9.0	E,I,P	4
Rhizoctoni solani	?	7.5 - 8.5	E	23
Sclerotia sclerotiorum	B	4.0	E,P	25
Sclerotium rolfsii	B	4.5	E,P	26
Thielaviopsis basicola	B	4.5 and 8.5	M,P	27
Uromyces phaseoli	A or B or C	4.0 - 5.0	P	16

[a] Abbreviations: E = from extracellular fungal culture; I = from intracellular fungal culture; M = from mixture of intracellular and extracellular; P = from infected plant tissue.

The properties of the phospholipase activities from cultures of P. infestans (4) are summarized in Table III. Of the five types of liquid media tested, the greatest phospholipase activity was obtained when P. infestans was grown in rye steep media. The phospholipase activity in rye culture filtrates was stimulated 15-fold by omitting glucose from the medium. The addition of 100 mg/liter of phosphatidylcholine (PC) to the rye medium caused an additional 35-fold stimulation in the activity of phospholipase. These experiments suggest that this enzyme activity is inducible. The addition of other lipids (sunflower oil, wax ester, and cholesterol oleate) to rye media also caused an apparent induction of phospholipase activity. We also detected extracellular phospholipase activity in lima bean agar cultures of P. infestans as shown in the second column of Table III. When the fungus was collected

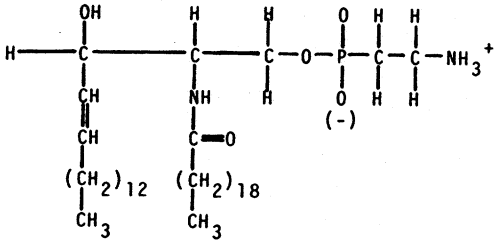

Figure 1. Ceramide aminoethylphosphonate (CAEP). When obtained from P. infestans the most common fatty acid is arachidonic acid and the most common long chain base is sphingosine.

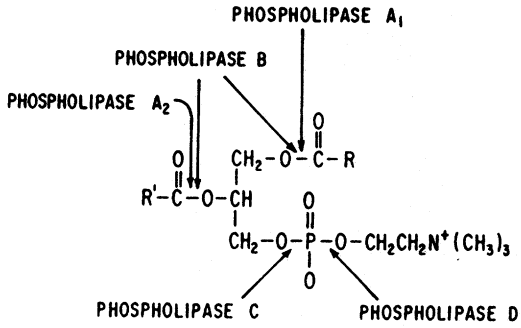

Figure 2. Sites of action of various types of phospholipases on phosphatidylcholine. Lysophospholipase hydrolyzes the carboxyl ester bond of a lysophospholipid (i.e., lysophosphatidylcholine).

Table III. A Summary of the Properties of the Phospholipase Activity from Cultures of P. infestans (4,7)

	Liquid Culture	Agar Culture
Media	Rye steep	Lima bean
Regulation	Repressed by glucose induced by PC	Not tested
Localization	Mostly extracellular	Extracellular
pH optimum	9.0	9.0
Effect of 5 mM DTT	96% Inhibition	94% Inhibition
Effect of 5 mM EDTA	None	55% Inhibition
Effect of 5 mM $CaCl_2$	5% Inhibition	17% Inhibition
Km for PC	2.86 μM	Not tested
Substrate specificity	TG $\simeq$ PC $\simeq$ MGDG	PC > TG

[a] Abbreviations: PC = phosphatidylcholine, TG = triacylglycerol, MGDG = monogalactosyldiglyceride, DTT = dithiothreitol, EDTA = ethylenediaminetetraacetic acid.

from liquid rye cultures (with added PC to induce phospholipase activity) much more phospholipase activity (30-fold) was found extracellularly than intracellularly. The pH optimum of the enzyme activity from both sources was 9.0. Dithiothreitol severely inhibited both enzyme activities. EDTA (5 mM) had no effect on the enzyme from liquid rye culture, but inhibited the enzyme from agar cultures. Both enzymes were slightly inhibited by 5 mM $CaCl_2$. A very low Km (2.86 μM) for phosphatidylcholine was measured using induced rye culture filtrate as a source of enzyme. When lipid substrates other than PC were tested the enzymes in the induced liquid culture were able to hydrolyze triacylglycerol (TG) and galactolipids (GL) at rates very similar to that for PC. In contrast, the enzymes from agar culture hydrolyzed PC at a rate about six times higher than for TG.

We have recently completed a study of the production of extracellular enzymes by germinating cysts of P. infestans (7). Although we were able to identify an esterase activity (p-nitrophenyl butyrate hydrolase) that appeared to be secreted during germination (0 to 20 h), phospholipase and lipase activities were apparently not secreted (Table IV). We are currently studying whether this esterase can hydrolyze any physiological substrates. This is an example of a case where the use of a nonphysiological substrate (PNP-butyrate) resulted in new and interesting information. However, extreme care must be exercised when trying to draw physiological conclusions from studies using nonphysiological substrates (i.e., p-nitrophenyl esters or 4-methylumbelliferyl esters). We are currently investigating a new phospholipase assay (28), which employs a fluorescent phospholipid substrate, 1-acyl-2-[6-[(7-nitro-2,1,3 benzoxadiazol-4-yl)amino]-caproyl] phosphatidylcholine(C_6-NBD-PC). Our preliminary studies (Table IV) indicate

Table IV. Changes in the levels of extracellular enzyme activities during germination (0 to 20 h) of cysts of P. infestans. The levels of the first three enzymes were previously reported (7). C_6-NBD-PC hydrolysis was measured as described (28).

Enzyme	Enzyme Activity n mol/min/10^6 Spores			
	0 h	5 h	10 h	20 h
p-nitrophenyl butyrate hydrolase	9.0	43.2	315.7	407.3
Lipase	.127	.132	.137	.138
Phospholipase (^{14}C-PC assay)	.590	.599	.604	.610
Phospholipase (C_6-NBD-PC assay)	.738	.735	.733	.732

that very similar values are obtained when phospholipase activity is measured with authentic phospholipid (^{14}C-PC) or the fluorescent phospholipid (C_6-NBD-PC). If it proves reliable, this new fluorometric assay will be much more convenient (30 assays per hour verses 15 assays per day using ^{14}C-PC).

Properties of the Phospholipase Activity in Healthy Potato Leaves

Many types of plant tissue contain high levels of lipolytic acyl hydrolase (LAH) activity (29). These enzymes are capable of hydrolyzing phospholipids (phospholipase B), galactolipids (galactolipase), and acyl glycerols. The LAH's in potato tubers and bean leaves have been purified and extensively studied (29). In 1979 Matsuda and Hirayama (30) reported that the total activity of phospholipase in potato leaves was about 400-fold lower than in potato tubers. They subsequently purified a lipolytic acyl hydrolase from potato leaves (31). The enzyme had a molecular weight of about 110,000 and a pH optimum of 5.0. The rate of hydrolysis of galactolipids was 7-fold higher than for phospholipids.

The following experiments illustrate that when studying the involvement of phospholipase in the host-pathogen interaction, the total contribution of enzyme of host origin may be considerably higher than previously realized. Rodionov and Zakharova (32) recently reported very high rates of autolytic hydrolysis of membrane lipids in homogenates of potato leaves (26-37% of the phospholipids were hydrolyzed after 2 h at 0-1°). Our laboratory recently confirmed this observation and proceeded to study some of the properties of the lipolytic acyl hydrolase activity in potato leaves (6). Lipolytic acyl hydrolase activity is apparently inactivated by polyphenol oxidase or its toxic quinone products.

When polyphenol oxidase activity was controlled, phospholipase activities ranged from 1.04 to 11.60 μ mol/min/gfw in the leaves of 41 North American cultivars (6). These values are much higher than those previously reported for potato leaves (.009 μmol/min/gfw) (30) and nearly as high as in potato tubers (2 to 30 μmol/min/gfw) (5,30).

Change in the Levels of Lipolytic Enzymes during Infection

Hoppe and Heitefuss (16) reported a 2-3-fold increase in phospholipase activity during the infection of susceptible bean leaves by U. phaseoli. During the infection of potato leaves by P. infestans we observed a greater than 14-fold increase in total phospholipase activity (Figure 3) (4). The increase in phospholipase activity was roughly proportional to the amount of leaf surface area that was covered by the fungus. When phospholipase activity was measured with the fluorescent substrate (C_6-NBD-PC), as previously described (28), it closely paralleled the curve obtained with ^{14}C-PC. However, when esterase activity was measured with another fluorometric substrate, 4-methylumbelliferyl laurate, it was highest at day 0 and decreased during infection. This indicates that 4-methylumbelliferyl laurate appears to be hydrolyzed by a lipolytic enzyme of the host.

To determine whether the increase in phospholipase activity during infection (Figure 3) was due to enzymes of fungal or host origin, the following experiments were performed. We observed that the pH optima of the phospholipase activity in fungal cultures was about 9.0 (Figure 4A,B) (4). Phospholipase activity was present in the uninfected leaves (Figure 4C), but it was much lower and exhibited optimum phospholipase activity at pH 6.0 as reported elsewhere (6). The infected leaf had a large peak of phospholipase activity at pH 8.0 to 9.0 and a smaller peak at pH 6.0. The two peaks of phospholipase activity in the infected leaves were further resolved by assaying in the presence of 5 mM DTT (Figure 4D) which is a potent inhibitor of fungal phospholipase. The peak of phospholipase activity at pH 9.0 was DTT-sensitive and likely to be of fungal origin. The peak of phospholipase activity at pH 6.0 was DTT-insensitive and is probably of host origin although further work is required to prove this beyond doubt.

Conclusions and Perspectives

These results indicate that during the infection of potato leaves by P. infestans, there is a rapid degradation of the host's membrane lipids (especially galactolipids) and a gradual increase in lipids of fungal origin (ceramide aminoethylphosphonate being the most unique). An analysis of phospholipase activity revealed that a DTT-sensitive phospholipase with an alkaline pH optima accumulated in infected leaves and was probably of fungal origin.

Although these preliminary studies indicate that lipolytic enzymes are produced by the fungus during infection, much more work is required to determine their actual role in infection and pathogenesis. Because the symptomology and biochemistry of infection are very similar to those events that occur during normal leaf

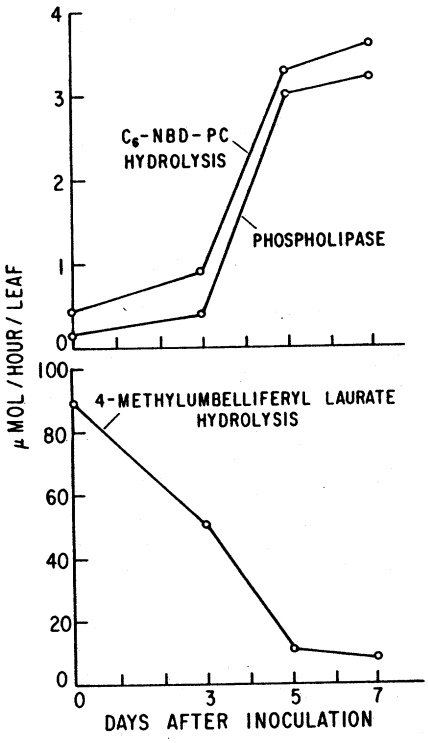

Figure 3. Time-course study of total phospholipase activity during infection of potato leaves by P. infestans. Phospholipase values were previously reported (4). C_6-NBD-PC hydrolysis assays were performed at pH 9.0 as described (28). Hydrolysis of 4-methylumbelliferyl laurate was measured by a fluorometric technique (44).

senescence (i.e., breakdown of chlorophyll, galactolipids, and protein), the two processes need to be very carefully compared (33). Both disease and senescence cause increases in membrane permeability (34,35). Recent work has shown that similar increases in the levels of free radicals, lipoxygenase activity, and lipid peroxidation occur during infection and senescence (36-38). Other studies indicate that under certain conditions phospholipid deesterification can be mediated by superoxide (O_2^-) in the absence of phospholipases (39). The most recent studies from our laboratory suggest that some of the lipolytic enzymes in potato leaves are regulated by calmodulin (40). Even if produced, free fatty acids may not accumulate in healthy or infected leaves because peroxisomes are capable of metabolizing fatty acids via β-oxidation (41). These factors all indicate that membrane lipid metabolism in healthy and diseased leaves is a dynamic process and any change in membrane composition needs to be interpreted very carefully.

Literature Cited

1. Thurston, H. D.; Schultz, O. In "Compendium of Potato Diseases"; Hooker, W. J., Ed.; American Phytopathological Society: St. Paul, 1981; p. 40.
2. Currier W. W. Trends in Biochemical Science 1981, 6, 191.
3. Bills, D. D. In "Host Plant Resistance to Pests"; Hedin, P. A., Ed.; ACS SYMPOSIUM SERIES No. 62, American Chemical Society: Washington, DC, 1977, p. 47.
4. Moreau, R. A.; Rawa, D. Physiol. Plant Path. 1984, 24, 187.
5. Moreau, R. A. J. Ag. Fd. Chem. 1985, 33, 36.
6. Moreau, R. A. Phytochemistry 1985, 24, 411.
7. Moreau, R. A.; Seibles, T. S. Can. J. Bot. (in press).
8. Wilson, U. E.; Coffey, M. D. Ann. Bot. 1980, 45, 81.
9. Shimony, C.; Friend, J. New Phytol. 1975, 74, 59.
10. Coffey, M. D.; Wilson, U. A. Can. J. Bot. 1983, 61, 2669.
11. Pitt, D.; Coombes, C. J. Gen. Microbiol. 1969, 56, 321.
12. Duddridge, J. A.; Sargent, J. A. Physiol. Plant Path. 1978, 12, 289.
13. Hoppe, H. H.; Heitefuss, R. Physiol. Plant Path. 1974, 4, 11.
14. Lösel, D. M. New Phytol. 1978, 80, 167.
15. Lösel, D. M.,; Lewis, D. H. New Phytol. 1974, 73, 1157.
16. Hoppe, H. H.; Heitefuss, R. Physiol. Plant Path. 1974, 4, 25.
17. Harwood, J. L. In "The Biochemistry of Plants: A Comprehensive Treatise"; Stumpf, P. K.; Conn, E. E., Eds.; Academic Press: New York, 1980; Vol. 4, p. 1.
18. Wassef, M. K.; Hendrix, J. W. Biochem. Biophys. Acta 1977, 486, 172.
19. Bostock, R. M.; Kuc, J. A.; Laine, R. A. Science 1982, 22, 67.
20. Faull, J. L.; Gay, J. L. Physiol. Plant Path. 1983, 22, 55.
21. Shepard, D. V.; Pitt, D. Phytochemistry 1976, 15, 1465.
22. Tseng, T. C.; Mount, M. S. Phytopathology 1974, 64, 229.
23. Tseng, T. C.; Bateman, D. F. Phytopathology 1968, 58, 1437.

24. Plumbley, R. A.; Pitt, D. Physiol. Plant Path. 1979, 14, 313.
25. Lumbsden, R. D. Phytopathology 1970, 60, 1106.
26. Tseng, T. C.; Bateman, D. F. Phytopathology 1969, 59, 359.
27. Lumbsden, R. D.; Bateman, D. F. Phytopathology 1968, 58, 219.
28. Wittenaur, L. A.; Shirai, K.; Jackson, R. L.; Johnson, J. D.; Biochem. Biophys. Res. Commun. 1984, 118, 894.
29. Galliard, T. In "The Biochemistry of Plants: A Comprehensive Treatise"; Stumpf, P. K.; Conn, E. E., Eds.; Academic Press: New York, 1980; Vol. 4, p. 85.
30. Matsuda, H.; Hirayama, O. Bull. Fac. Agric. Shimane Univ. 1979, 13, 105.
31. Matsuda, H.; Hirayama, O. Biochim. Biophys. Acta 1979, 573, 155.
32. Rodionov, V. S.; Zakharova, L. S. Soviet Plant Physiol. 1980, 27, 298.
33. Novacky, A. In "Biochemical Plant Pathology"; Callow J. A., Ed.; John Wiley and Sons: Birmingham, U.K., 1983; p. 347.
34. Wheeler, H. In "Plant Disease: An Advanced Treatice"; Horsfall, J. G.; Cowling, E. B., Eds.; Academic Press: New York, 1978; Vol. 3, p. 327.
35. Barber, R. F.; Thompson, J. E. J. Exp. Bot. 1980, 31, 1305.
36. Lupu, R.; Grossman, S.; Cohen, Y. Physiol. Plant Pathol. 1980, 16, 241.
37. Dhindsa, R. J.; Plumb-Dhindsa, P.; Thorpe, T. A. J. Exp. Bot. 1981, 32, 126.
38. Thompson, J. E.; Pauls, K. P.; Chia, L. S.; Sridhara, S. In "Biosynthesis and Function of Plant Lipids"; Thomson, W. W.; Mudd, J. B.; Gibbs, M., Eds. American Society of Plant Physiologists: Rockville, MD, 1983; p. 173.
39. Niehaus, W. J., Jr. Bioorg. Chem. 1978, 7, 77.
40. Moreau, R. A.; Isett, T. Plant Science (in press).
41. Gerhardt, B. FEBS Letts. 1981, 126, 71.
42. Hara, A.; Radin, N. S. Anal. Biochem. 1978, 90, 420.
43. Christie, W. W. "Lipid Analysis"; Pergamon Press: Oxford, U.K., 1982.
44. Hasson, E.P.; Laties, G. G. Plant Physiol. 1976, 57, 142.

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